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Removal of *Clostridium perfringens*, *Escherichia coli* and F-RNA coliphages by stormwater biofilters

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ABSTRACT

Biofiltration is a technology applied to treat urban stormwater runoff that transports various pollutants, including pathogens. However, the pathogen removal performance of biofiltration systems remains unknown. A laboratory study was conducted to investigate the removal of three indicator organisms (*Clostridium perfringens*, *Escherichia coli*, and F-RNA coliphages) by biofilters. The influence of a range of factors was investigated: presence of vegetation, depth and types of filter media, presence of a saturated zone at the base of the biofilter, and intermittent wetting and drying conditions. The mean removal of *C. perfringens* and F-RNA coliphages by all biofilter designs exceeded 3 log. *E. coli* removal during wet periods, however, was much lower (mean 2 log). Furthermore, antecedent drying decreased the *E. coli* removal efficiency significantly ($p < 0.05$). Drying might induce fine fissures or macropore formation in filter media thus reduced retention of microbes. This effect may be more obvious in vegetated designs due to evapotranspiration induced moisture loss. Introducing a saturated zone and carbon source at the base of the filter eliminated such negative effects of drying on *E. coli* removal. The effluent from biofilters with a saturated zone and carbon source met the recommended water quality for secondary contact recreational water use in relation to *E. coli*.

1. Introduction

Untreated urban stormwater runoff has frequently been identified as a nonpoint source of pathogens for many receiving water bodies (USEPA, 2005; Collins et al., 2010), with the potential to adversely affect drinking water sources, recreational waters and aquaculture. Adverse health effects, even waterborne diseases in human populations, have been associated with contacting such contaminated water bodies (Haile et al., 1999; Gaffield et al., 2003). Consequently, urban stormwater management, particularly reduction of pathogen levels, has drawn extensive attention.

For ease of water quality management, *Clostridium perfringens* spores (*C. perfringens*), *Escherichia coli* (*E. coli*), and F-RNA coliphages are commonly used indicators for protozoan, bacterial, and viral pathogens respectively (Ferguson et al., 2003). All these organisms are relatively safe to use and easily detected, thus presenting affordable methods of understanding pathogen behaviour. More importantly, these organisms have shown close relationship with their indicated pathogens in terms of presence, survival, and rare extensive growth in aquatic or soil environment. The main criticism of the use of *C. perfringens* spores as a faecal indicator is its prolonged persistence in the environment (Medema et al., 1997). However, this is one of the reasons it is often used as a conservative indicator for the presence and fate of protozoa in aquatic systems (Payment and Franco, 1993). *E. coli* is a commonly used indicator for assessing overall water quality. F-RNA coliphages were chosen as viral indicators, since they behave relatively conservatively, and have been shown to be very persistent. Moreover, they resemble human enteric viruses in size, shape and composition (Havelaar et al., 1991, 1993; Schijven and Hassanizadeh, 2000).

Biofilters, also known as bioretention systems or rain gardens (Fig. 1), are employed globally to treat urban runoff prior to discharge. Typical biofilters are a combination of natural and engineered systems, which work by filtering stormwater through vegetated filter media that removes pollutants by means of biological uptake, straining and adsorption (Rusciano and Obropta, 2007). Biofilters have been designed and thoroughly studied to remove typical pollutants from stormwater, such as sediments, nutrients

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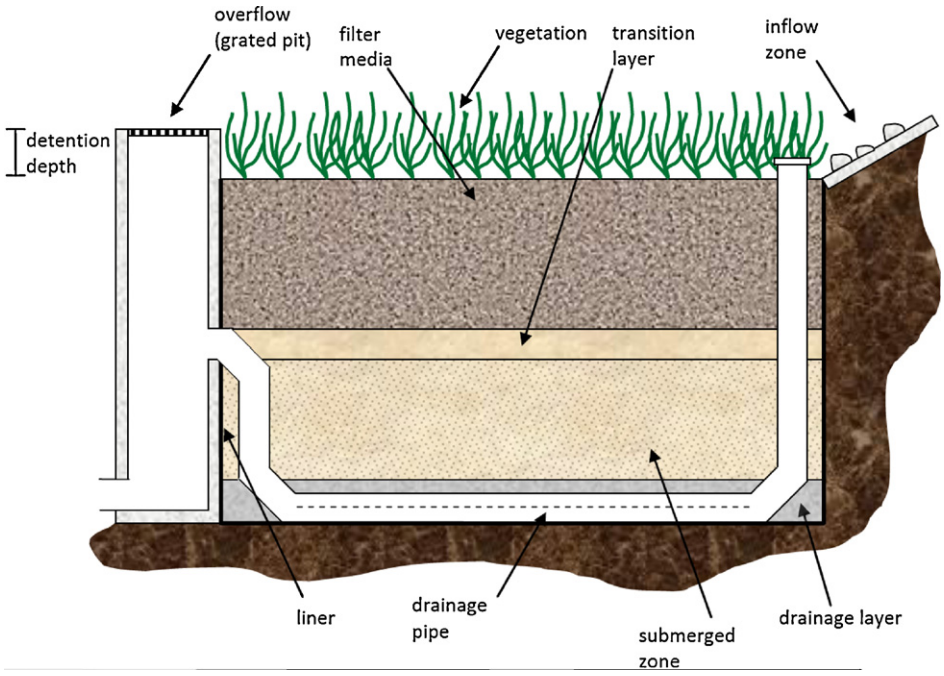

Fig. 1. Biofilter design with saturated zone as recommended by Facility for Advancing Water Biofiltration (FAWB, 2009).

and metals (Davis et al., 2001; Dietz and Clausen, 2006; Dietz, 2007; Fletcher et al., 2008). Yet, their capacity to remove indicator organisms has rarely been reported.

In several feasibility studies, effective microorganism removal from stormwater has been shown, with *E. coli* and faecal coliforms removal in excess of 90% (Rusciano and Obropta, 2007; Hathaway et al., 2009; Chandasena et al., 2011). Even in non-vegetated biofilters, *E. coli* removal has been shown to be over 90% with the majority of bacteria being entrapped in the top layer of the filter media (Zhang et al., 2011). Thus, the filter media is the most important biofilter engineered component regarding microorganism removal. Furthermore, plants in biofilters can interfere with microbial removal performance by facilitating growth of beneficial bacteria, adsorption and predation of pathogens in the region of the soil under the immediate influence of living roots, termed rhizosphere (Bitton and Marshall, 1980; Rusciano and Obropta, 2007). In addition, plant root growth and die-off can help maintain hydraulic conductivity (Hatt et al., 2009), whereas the decayed roots may produce macropores or channelling in the media resulting in reduced filtration capacity of the filter media.

Stormwater events are highly variable in frequency, exposing biofilters to intermittent wetting and drying conditions. Drying might affect the structure and function of biofiltration system, e.g. fine fissures and macropores formation in filter media, preferential flow along dying roots, decreased plant activity, decreased biofilm activity (Blecken et al., 2009a). In addition, the level of moisture content in filter media can influence the survival of trapped microbes within the media (Schijven and Hassanizadeh, 2000; Stevik et al., 2004). However, the effect of this variability on microbial treatment by biofilters has regrettably been ignored. In addition, since viruses and protozoan differ from bacteria in survival, surface properties and size, their fate and transport in biofilters is expected to differ from that of bacteria. Hence, feasibility assessment and optimisation of removing viruses and protozoan by biofilters is a necessity.

This paper presents a laboratory study of biofilters at removing protozoan, bacterial, and viral indicators under intermittent wetting and drying conditions. The impact of several key biofilter design parameters including vegetation, filter media type, filter media depth and a saturated zone was investigated. The aims of the work were:

- To assess protozoan (*C. perfringens* spores), bacterial (*E. coli*), and viral (F-RNA coliphages) indicators removal by biofilters under the provision of regular stormwater inflow.
- To determine the influence of extended drying upon three indicators removal during subsequent storm events.
- To determine the optimal biofilter design for removal of three indicator organisms.

2. Materials and methods

2.1. Experimental set-up

28 biofilter columns were constructed from 375 mm diameter PVC pipes, with a transparent Perspex top section allowing for plant growth and ponding of water (Fig. 2). The inner walls of the filters were sand blasted in order to minimise preferential flow effects. Mesocosms were tested in a specially constructed greenhouse with a clear impermeable roof admitting full, natural sunlight.

To assess the influence of design parameters on indicator organisms removal, we tested six biofilter configurations using alterations of current biofilter design parameters (Table 1). 'Standard' biofilters consisted of 700 mm deep sandy loam (with a d_{50} of 0.25 mm) planted with *Carex appressa* which is commonly used in biofilters since it is instrumental for good removal of nutrients (Bratieres et al., 2008; FAWB, 2009). Sandy loam was used as the primary filter media for all configurations with basic characteristics shown in Table 2. 'Non-vegetated' biofilters ('Unveg') and 'Short' biofilters were created to determine the influence of vegetation and filter media depth on indicator organisms removal respectively. The same sandy loam media, mixed with 20% vermiculite and perlite ('SLVP' biofilters), was also trialled in this study, since it was shown to improve the removal of other pollutants related to human health risks (especially heavy metal removal because of the

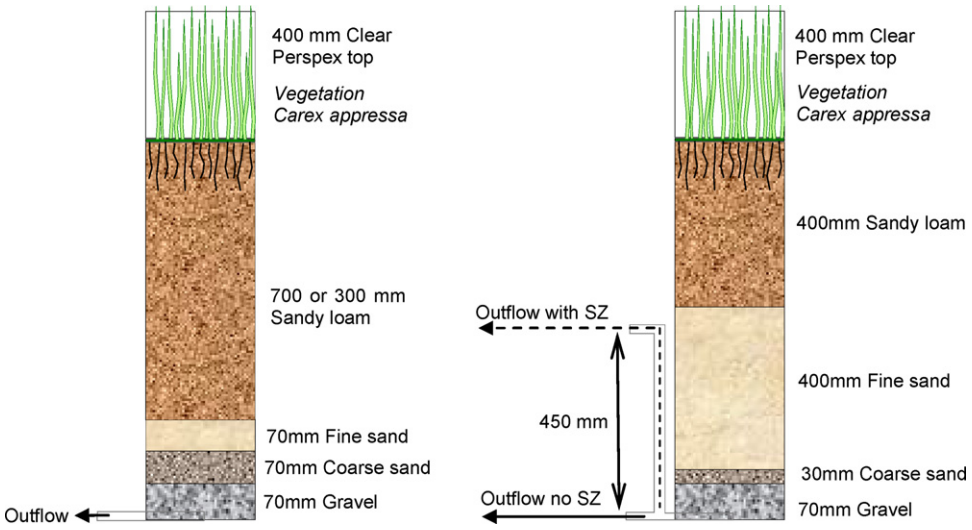

Fig. 2. Construction details of standard biofilters (left) and advanced biofilters (right).

Table 1
Biofilter configurations.

Configurations	Design characteristics				
	Vegetation	Media depth (mm)	Media type[a]	SZ[b]	Replicates
Standard	*Carex*	700	SL	None	5
Unveg.	None	700	SL	None	5
Short	*Carex*	300	SL	None	5
SLVP	*Carex*	700	SLVP	None	5
Advanced	*Carex*	400	SL	None	4
C+SZ	*Carex*	400	SL	SZ	4

[a] SL, sandy loam; SLVP, sandy loam mixed with 10% vermiculate and 10% perlite.
[b] SZ, saturated zone with carbon source

increased cation exchange capacity) (Hatt et al., 2007). 'Advanced' biofilters had a layer of sandy loam of only 400 mm, and an additional layer of fine sand of 400 mm just underneath (Fig. 2). In 4 of the 'advanced' biofilters, a saturated zone (SZ) of 450 mm was established by elevating the pipe outlet (Fig. 2). In the SZ, a cellulose carbon source was added in the sand (800 g consisting of 2/3 redgum wood chips and 1/3 pea straw) (Blecken et al., 2009b). These were 'C+SZ' biofilters, which are used in practice for enhancing nitrogen removal (Dietz and Clausen, 2006).

After about 4 months of plant establishment, each column was dosed with 25 L 'semi-natural' stormwater (see Bratieres et al., 2008 for details on the water qualities) twice weekly for 11 months prior to this microbial removal test commencing. This dosing regime simulated what a biofilter sized to 2% of its impervious catchment area would receive on average in Melbourne; Melbourne has an average annual rainfall of 653 mm (Bratieres et al., 2008). As a result, the columns used in this study were mature systems that had an average hydraulic conductivity of 115 mm/h. This value is within the recommended range for practice (FAWB, 2009). We can therefore surmise that the mesocosms were well established with microbial and plant communities, as they would be in typical operation conditions.

Table 2
Water quality of the inlet of Ruffey's Creek wetland and geochemical characteristics of the sandy loam filter media.

Water quality			Sandy loam characteristics	
Parameter	Urban stormwater[a]	Ruffey's Creek[b]	Parameter	Sandy loam
TSS (mg/L)	19–254	NA	pH	8.2
TP (mg/L)	0.08–1.26	0.35–0.65	EC (μS/cm)	57
TN (mg/L)	0.6–7.5	2.6–2.9	TOC (mg/kg)	12,000
pH	5.5–7.3	7.0–7.4	CEC (mequiv./100 g)	18
EC (μS/cm)	–	1000	Gravel (>2 mm, %)	1
Pb (mg/L)	0.02–0.16	NA	Grain size (d_{50}, mm)	0.25
Zn (mg/L)	0.08–0.57	NA	d_{60}/d_{10}	4.4
Cu (mg/L)	0.01–0.14	NA	Silt and clay (%)	8
C. perfringens (cfu/100 ml)	100–3000	0–680		
F-RNA coliphages (pfu/100 ml)	3000	<1–1500		
E. coli (MPN/100 ml)	3500–185,000	2200–15,000		

[a] Urban stormwater quality except for F-RNA coliphages taken from (NRMMC et al., 2009); F-RNA coliphages taken from measurements the authors made in five urban catchments in Melbourne, Australia (unpublished data); NA – not assessed since this had been measured previously and found to be within acceptable levels.
[b] Ruffey's Creek taken from pilot sampling campaigns.

2.2. Experimental procedure

2.2.1. Dosing

The first 2.5 weeks of dosing corresponded to an establishment period to inoculate the columns. Next, wetting and drying dosing schemes ensued in the following order: 2 weeks wet/2 weeks dry/3.5 weeks wet/2.5 weeks dry/2 weeks wet. During wet periods, each column was dosed twice weekly, whereas during dry periods they received no inflow. On dosing days, natural stormwater was extracted from the inlet of Ruffey Creek Wetland (a medium density residential development in Melbourne with total catchment area of 106 ha) with sediments well disturbed to ensure that suspended solids were pumped with the water. The collected stormwater had reasonable levels of *E. coli* but lower levels of the other two indicator organisms (Table 2). In addition, all measures were taken to minimise significant changes to its quality during pumping, transport, and dosing. Each column was dosed with 25 L stormwater in 5× 5 L "pulses" to ensure that any variation over time in the concentration would be consistent for all columns.

2.2.2. Sampling

C. perfringens, *E. coli*, and F-RNA coliphages were selected as indicator organisms for protozoa, bacteria, and viruses respectively.

Five sampling runs were undertaken during the 3.5 months of dosing. Sampling Runs 1, 3 and 5 were conducted on the last dosing day of wet periods, while Sampling Runs 2 and 4 were conducted on the first dosing day after dry periods. This sampling regime enabled us to assess the impact of the intermittent wetting and drying conditions. On sampling days, the same methods as for "dosing days" were followed to collect stormwater and dose the biofilters except that the collected stormwater was spiked with lab cultures of the three indicator organisms supplied by a NATA-accredited laboratory. The spiking was targeted to be in the range typically found in urban stormwater (Table 2). In Sampling Run 1, however, the stormwater was unintentionally spiked with unusually high concentration of *E. coli* and F-RNA coliphages, which can be regarded as a test of biofilters under challenging conditions (e.g. sewage cross connection). The other four sampling runs have *E. coli* concentrations within 95th percentile of *E. coli* level in urban catchments (NRMMC, EPHC and NHMRC, 2009). The spiking and mixing was at least 30 min before dosing commencing to allow microorganisms and particles to reach equilibrium interactions. Although we have tried to mimic the field conditions by dosing with natural stormwater (that contained in situ sediments) that was spiked with three pathogen indicators on site, it is still possible that there are some differences regarding particle/microbe interaction, between our spiked stormwater and stormwater that naturally contains the given levels of organisms.

An attempt was made to capture Event Mean Concentration of the inflows and outflows from each column. Sampling Run 1 proceeded as follows: one 1 L composite inflow sample was taken, consisting of five 200 ml sub-samples taken after every 5 L of stormwater dosed in the column. The 1 L composite outflow for each column was also made up of five sub-samples: the first sub-sample was collected after 1 L effluent flowed out, followed by four other subsamples, each after 5 L effluent. During the next four sampling runs, the outflow sampling was modified; each subsample was collected after every 1 L effluent. This was done to ensure that the samples could be delivered to the laboratory for analysis within 6 h and avoid complications such as regrowth, die-off, etc. The microbial analysis of all the samples was conducted by a NATA accredited laboratory using standard methods. The *C. perfringens* spores test was done by Membrane Filtration (Australian/New Zealand Standard™, 2000), *E. coli* by Defined Substate Technology (Australian Standards™, 2005), and F-RNA coliphages by USEPA 1602/APHA 9224 (USEPA, 2001).

The moisture content in the biofilter top layer (at 225 mm below the soil surface) was continuously measured (ML2x ThetaProbe, Delta-T Devices Ltd., UK) for 3 'advanced' biofilters and 3 'C+SZ' biofilters. The moisture data measured at the beginning of each sampling run was used to determine the impact of moisture content on the removal of indicator organisms.

The time required for the first 6 L stormwater to pass through the columns during Sampling Runs 3, 4, and 5 was measured. This was applied as an indicator of the detention time.

2.3. Data analysis

All indicator organisms concentrations below their respective detection limits were replaced by a value equal to that of the detection limits. Geometric mean concentrations of microbial indicators in inflow and outflow from each configuration were calculated (the values were rounded to significant digits). Log removal values were calculated based on log concentration differences between inflow and outflow samples. The distributions of inflow concentrations, outflow concentrations, and the log removal rates for each configuration over each sampling run, were presented in the form of boxplots. All indicator organisms data were checked for normality using the Shapiro–Wilk test. Non-parametric analysis (Kruskal–Wallis test) was used in cases where the assumption of homogeneity of variance could not be met even after log transformation of the data. Following a significant finding (i.e. if $p < 0.05$), post hoc tests were then performed, using either the Kruskal–Wallis Z test (applying non-parametric analysis) or the Tukey test (applying parametric analysis). Linear regression tests were used for correlation analysis. For example, log-transformed inflow concentration versus log-transformed outflow concentration, log removal versus detention time, and log removal versus moisture content were analysed.

3. Results and discussion

Table 3 shows geometric mean inflow and outflow concentrations and logarithmic reduction efficiency of 6 biofilter configurations over 5 sampling runs for *C. perfringens*, *E. coli* and F-RNA coliphages. In general, the outflow concentrations of the three microbial indicators were significantly lower than the inflow concentrations for each biofilter design ($p < 0.05$). For both *C. perfringens* and F-RNA coliphages, over 3 log reductions were achieved; for *E. coli*, the reduction (1.6 log with a standard deviation of 1.0 log) was significantly lower ($p < 0.05$), and varied with biofilter configurations. Among the 6 biofilter designs, only filters with saturated zone and carbon source ('C+SZ') had a mean outflow concentration of *E. coli* (450 MPN/100 ml) lower than the recommended water quality designated for secondary contact recreational water use, i.e. median 878 *E. coli*/100 ml (ANZECC/ARMC, 2000). A detailed analysis was conducted, investigating the influence of the chosen design and operational conditions on the removal of each indicator.

3.1. Clostridium perfringens spores

C. perfringens spores were effectively removed by all biofilters configurations, despite intermittent wetting and drying conditions: a mean log removal of 3.1 was achieved. In fact, over 90% of the outflow samples had *C. perfringens* spores concentrations below 10 cfu/100 ml in lieu of inflow concentrations 1000–19,000 cfu/100 ml (Fig. 3). High variability was observed in

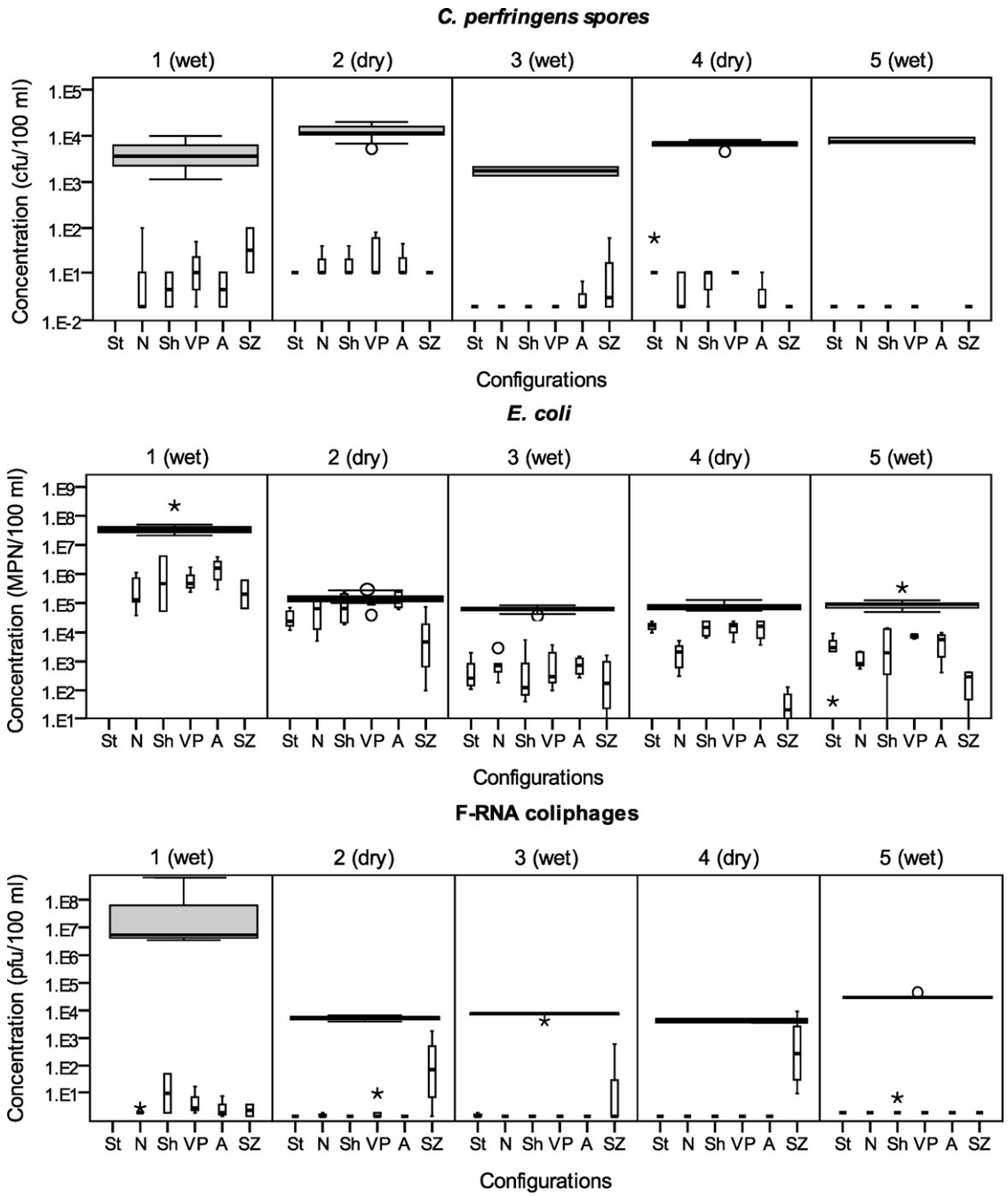

Fig. 3. Range of outflow (open sub-boxplots) and inflow (shaded grouped-boxplots) concentrations of *C. perfringens*, *E. coli* and F-RNA coliphages over 5 sampling runs relative to 6 biofilter configurations. St, standard; N, Unveg.; Sh, Short; VP, SLVP; A, advanced; SZ, C+SZ (note that results for 'standard' biofilters were not available in Run 1 due to sampling problems).

Sampling Run 1. This variability diminished over time (Fig. 3), indicating a subtle, positive impact of the system's maturity.

Straining and attachment are hypothesised to be the key removal mechanisms for *C. perfringens* spores in the studied filters. A high degree association of *C. perfringens* spores with settleable particles in stormwater, particularly at high particle concentrations, i.e. 100–200 mg/L as would be in typical stormwater was observed in (Characklis et al., 2005), which may allow efficient removal via sedimentation and physical straining by soil columns in this study. Attachment of *C. perfringens* spores to filter media through hydrophobic and electrostatic interactions has also been verified to contribute to their retention in sand filters (Schijven et al., 2003). On the other hand, predation and natural die-off were proven to be ineffective for *C. perfringens* removal in sediments (Davies et al., 1995), which may indicate that they have played a minor role in the observed removal performance. Both straining and attachment could be enhanced by clogging of filtration systems (Stevik et al., 2004), but this is rather unlikely to affect the studied biofilters, since the filters used in this study were already mature and their hydraulic conductivity were shown to not change significantly over time; this is in agreement with previous studies that suggest that matured (and well-designed) systems maintain their hydraulic performance due to plant root activity (Hatt et al., 2009; Le Coustumer et al., 2009).

Table 3
Geometric mean concentrations, log reductions for *C. perfringens* spores, *E. coli* and F-RNA coliphages for 6 configurations.

Configurations	*C. perfringens* spores			*E. coli*			F-RNA coliphages		
	Concentration (cfu/100 ml)		Log removal	Concentration (MPN/100 ml)		Log removal	Concentration (pfu/100 ml)		Log removal
	Inflow	Outflow		Inflow	Outflow		Inflow	Outflow	
Standard	6300	4	3.20	91,000	4600	1.30	8000	1	3.90
Unveg.	4900	3	3.21	390,000	5800	1.83	38,000	1	4.58
Short	5800	3	3.29	200,000	6000	1.52	16,000	1	4.20
SLVP	5100	6	2.93	250,000	14,000	1.25	19,000	1	4.28
Advanced	4500	3	3.18	330,000	22,000	1.18	48,000	1	4.68
C+SZ	4600	4	3.06	190,000	**450**	2.63	15,000	1	3.13
Total	5200	4	3.11	230,000	5700	1.61	21,000	11	4.32

Note: cfu, colony-forming units; MPN, most probable number; pfu, plaque-forming units.
Bold value indicates two significant figures.

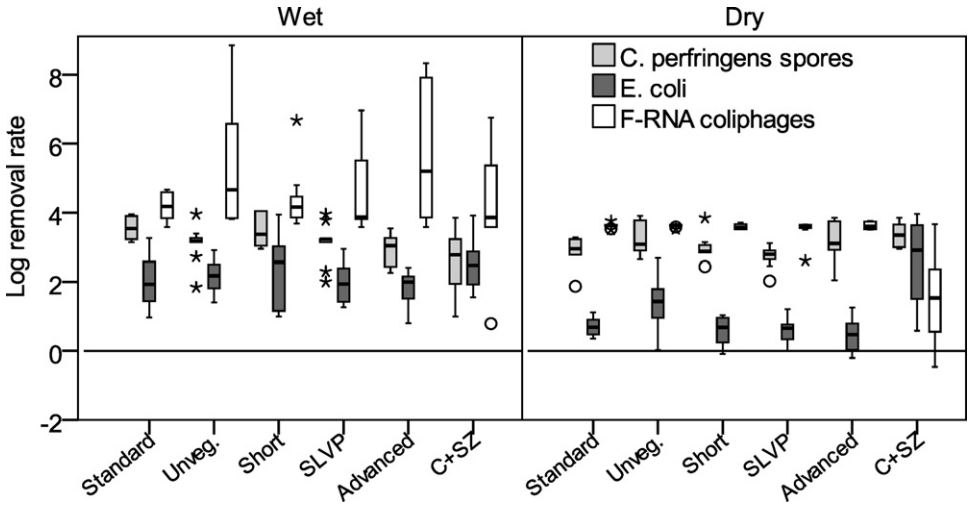

Fig. 4. Log removal of *C. perfringens*, *E. coli* and F-RNA coliphages relative to the biofilter configurations and the wet (Sampling Runs 1, 3, and 5) and dry (Sampling Runs 2 and 4) periods.

3.2. *Escherichia coli*

E. coli removal efficiency of biofilters was lower and more variable than that of *C. perfringens*, with some configurations even yielded a net increase in *E. coli* effluent concentrations, as in the case of 'short' filters and 'advanced' filters (Figs. 3 and 4). A linear regression test identified a positive trend between log transformed *E. coli* inflow and outflow concentrations (Sampling Run 1, however, was eliminated from this test due to an extremely high inflow concentration of *E. coli*) (Fig. 5) indicating that the *E. coli* retention efficiency of the biofilters was not affected by fluctuations in inflow concentrations. Whilst this was not found for *C. perfringens*, such a trend is commonly identified in stormwater treatment systems (Davis et al., 2006).

3.2.1. *Configuration type*

Configuration type was not a significant factor for *E. coli* removal during wet periods (Sampling Runs 1, 3, and 5) ($p > 0.05$) (Figs. 3 and 4). An average log removal of 2.0 was achieved despite various column designs. Comparable *E. coli* removal performance of biofilters has been demonstrated before (Hathaway et al., 2009; Zhang et al., 2011). Improvement of removal efficiency over time as observed in (Rusciano and Obropta, 2007; Kadam et al., 2008; Zhang et al., 2011) was not obvious in this study, partly because the columns used herein were mature systems and experiment duration was limited, i.e. only 3.5 months.

Straining was hypothesised to be an important process for reducing *E. coli* effluent concentrations in biofilters. The association of *E. coli* with settleable particles in natural stormwater (Characklis et al., 2005) can facilitate sedimentation and straining processes for *E. coli* removal by filtration systems. In addition, biofilm formed in these mature systems may contribute to decreased porosity, along with increased dispersion (Zhang et al., 2011), thus enhanced straining efficiency. The importance of attachment for

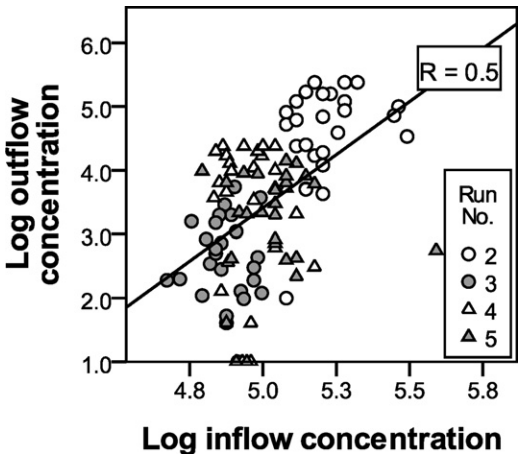

Fig. 5. Log outflow concentration of *E. coli* relative to log inflow concentration.

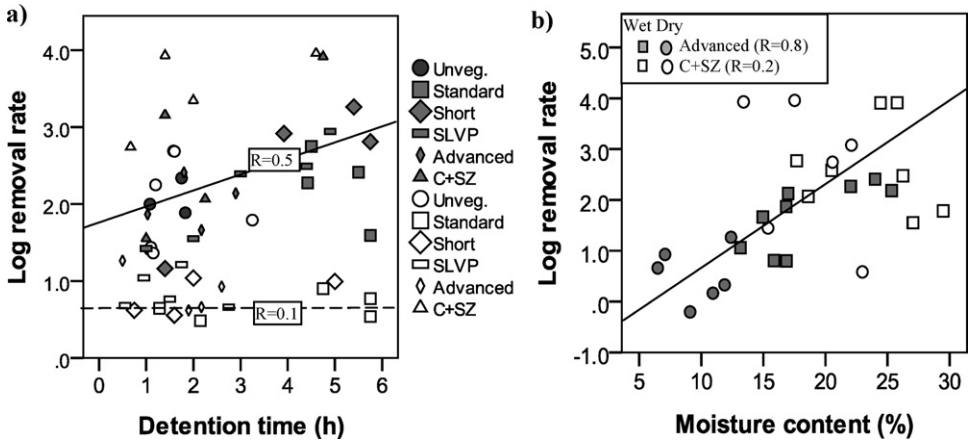

Fig. 6. Log removal rate of *E. coli* relative to (a) detention time and biofilter configurations over Sampling Run 3 (shaded markers) and Run 4 (open markers); (b) moisture content and biofilter configurations over 5 sampling runs.

E. coli removal in soil filter media should be highlighted. A positive correlation between *E. coli* removal efficiency and detention time was identified: better removal efficiency at higher detention time (Sampling Run 3; $p < 0.05$; Fig. 6a). Field results are similar to those observed herein (Hathaway et al., 2011). In general, low detention time indicates shortened contact, thus reduced utilisation of surface area and decreased adsorption (Bitton and Marshall, 1980; Stevik et al., 2004). In addition, low detention time also meant high flow rate, therefore more microbial movement and less straining. Yet in the analysis of each configuration type, this trend was not always observed (Fig. 6a). This might be due to the low number of replicate columns used to investigate these types of trends (i.e. each configuration only had four to five data points): as such, more studies are required. The observed lower *E. coli* removal rate in these biofilters than that of *C. perfringens* could be due to the accumulation and release of indigenous *E. coli*, since the columns were dosed twice weekly with natural stormwater having a much higher level of indigenous *E. coli* (2200–15,000 MPN/100 ml) than *C. perfringens* (0–680 cfu/100 ml, Table 2). Another explanation is the lesser extent of association of *E. coli* with settleable particles (20–55%) than *C. perfringens*, therefore less straining efficiency.

3.2.2. Wetting/drying regime

Wetting/drying regime had a significantly negative impact on *E. coli* removal by vegetated filters without SZ ($p < 0.05$) (Fig. 4): adequate removal after wet periods (1.9 log); yet low and variable removal rates after dry periods (0.6 log). Interestingly, non-vegetated biofilters after a long dry period (Sampling Run 4) outperformed most other configurations without SZ. The introduction of an SZ and carbon source in biofilters maintained optimal *E. coli* removal after antecedent drying periods (Figs. 3 and 4): effluent *E. coli* concentrations from 'C+SZ' were around 610 MPN/100 ml after wet periods and 310 MPN/100 ml after dry periods; *E. coli* removal was 2.7 log after wet periods and 2.6 log after dry periods.

Extended drying resulted in a decrease in soil moisture content in vegetated biofilters without SZ. For example, a 50% decrease in soil moisture content was measured in 'advanced' columns following 2–2.5 weeks drying (Fig. 6b). However, biofilters with an SZ and carbon source maintained moist conditions throughout the drying periods.

It is likely that the reduced soil moisture content – following extended dry periods – results in increased porosity and less optimal functioning of biofilm. This was confirmed by detention time measurement: an obvious decrease in detention time after a dry period (Sampling Run 4) was observed, particularly in 'standard' columns, 'short' columns, and 'SLVP' columns (Fig. 6a) alongside a manifestly delayed flow at the commencement of dosing. This therefore might reduce the removal of *E. coli* by these configurations as a consequence of mobilisation of fine sediments, detachment of accumulated *E. coli* from former events, less extent of contact between water and filter media, and possibly less straining of particulate-bound *E. coli*. Furthermore, reduced moisture content can result in greater opportunity of creation of fissures traversing the entire depth of the media in columns with reduced filter media depth, evidenced by negative removal by 'short' columns and 'advanced' columns in Sampling Run 2 (after a dry period). Reduced removal of heavy metals following extended dry periods was observed in a previous study (Blecken et al., 2009b).

The absence of vegetation delayed the aforementioned negative effect, perhaps due to less moisture loss than in vegetated columns with pronounced plant transpiration. In fact, detention time and log removal by non-vegetated biofilters after an antecedent dry period of 2.5 weeks (Sampling Run 4) remained similar to those found in Sampling Run 3. Hatt et al. (2007) also observed no significant influence of the wetting and drying conditions on phosphorous and metal removal by unvegetated biofilters.

The introduction of an SZ and carbon source eliminated the effect of drying on *E. coli* removal (Figs. 3 and 4). Furthermore, the high moisture content (Fig. 6b) and carbon source in 'C+SZ' biofilters may prolong the survival of predators and competing microbes, resulting in faster die-off for introduced bacteria as evidenced by (Postma and Vanveen, 1990). In addition, a more extensive root system might exist in these columns due to the consistently high moisture content, which demands further investigation. This could therefore enhance the microorganism adsorption in the region of the soil under immediate influence of living roots, due to the presence of energy-rich and growth-promoting organic compounds (Bitton and Marshall, 1980). More importantly, the proportion of antecedent water in the outflow was high in 'C+SZ' biofilters, resulting in an effectively longer average detention time. Nevertheless, it is also possible that the cells enter a viable but non-culturable state, rendering them undetectable by the Colilert method (Davies et al., 1995).

3.3. F-RNA coliphages

F-RNA coliphages are the smallest of the three indicators tested (21–30 nm), yet they exhibited comparable log removals to *C. perfringens* and better removals than *E. coli* (Table 3, Figs. 3 and 4). In fact, with the exception of biofilters with SZ, the outflow

concentrations of F-RNA coliphages remained relatively low; for example, over 85% of the outflow samples had concentrations equal to, or below, the detection limit (1 pfu/100 ml), whereas the inflow concentrations were 10^3–10^9 pfu/100 ml (Fig. 3).

3.3.1. Configuration type

Configuration type again played an insignificant role in virus removal after wet periods ($p > 0.05$) (Figs. 3 and 4). 'C+SZ' biofilters performed similarly to other configurations, with the exception of just one of the replicates during Sampling Run 3, which could have been caused by sampling errors, since this replicate produced an outlet concentration of 611 pfu/100 ml, while the other three replicates managed to remove all F-RNA coliphages. Mean F-RNA coliphages reduction efficiency of 4.8 log was achieved over all biofilters, which was higher than that of MS2 (a prototype F-RNA coliphages) removal by Castricum soil columns (Hijnen et al., 2005).

Both attachment and straining were supposed to be significant processes in F-RNA coliphages removal by our filters. Hijnen et al. (2005) demonstrated that attachment was the most important process for MS2 removal by soil columns. The higher ionic strength in the stormwater, higher organic matter and cation exchange capacity in sandy loam (Table 2), may have collectively enhanced the attachment in these filters. In addition, as mentioned earlier, the filters used in this study were mature systems in which there was surface clogging, i.e. an accumulation of solid particles on the top surface of the columns as observed in a similar study (Hatt et al., 2008) and biofilm formation. Biofilm could enhance the attachment process through increasing polymeric interactions between the biofilm and the virus particles, increasing media surface roughness, reducing hydraulic conductivity and increasing dispersion (Stevik et al., 2004). Biofilm formation can also enhance straining due to reduced porosity and improved dispersion (Zhang et al., 2011). In fact, 20–60% coliphages have been shown to associate with settleable particles in stormwater (Brookes et al., 2005; Characklis et al., 2005), making them easier to be removed via straining.

3.3.2. Wetting/drying regime

All the configurations without SZ performed with no obvious difference during dry-wet events ($p > 0.05$). That is, decreased removal rate due to the reduced moisture content after dry events in biofilters without SZ was not observed for F-RNA coliphages. One explanation is that less moist environments allow air to fill the empty spaces, and air–water interface has been shown to significantly retain and/or inactivate viruses (Schijven and Hassanizadeh, 2000).

However, biofilters with SZ ('C+SZ') configuration was negatively affected by the wetting/drying regime ($p < 0.05$) (Figs. 3 and 4). This could indicate that a SZ (i.e. high moisture conditions) lowered the inactivation rate of F-RNA coliphages. F-RNA coliphages may remain active in the filter media and be eluted during the subsequent dosing session or sampling run. This effect is hypothesised to be more obvious following dry periods, because the filter media before a dry weather sampling run was primed with a high level of F-RNA coliphages, which was spiked during the preceding sampling run. The infection of *E. coli* by F-RNA coliphages (Meyvisch et al., 1974; Havelaar et al., 1991) might be another explanatory factor. Although, such interaction is rare in water environments due to low concentrations, it might occur when they are concentrated through straining and adsorption in soil columns. If so, these phages could survive or even grow in the infected host bacteria and then be remobilised during the subsequent rain event. Based on limited data, detention time seems to impact the treatment of F-RNA coliphages in 'C+SZ' configurations, especially after dry periods: the higher the detention time, the better the removal rate. It is suggested that the 'C+SZ' columns with higher detention time may exert limited water transport and limited detachment of viruses, thus greater retention.

4. Implications of biofilter design and operations on pathogen removal

The application of biofilters for pathogen removal from stormwater was explored through assessing the effectiveness of biofilters at removing protozoan (*C. perfringens* spores), bacterial (*E. coli*), viral (F-RNA coliphages) indicators. However, due to the differences between pathogens and indicator organisms, the findings discussed below should be applied with caution.

Biofilters are promising techniques for urban stormwater management. Both protozoa and viral indicators were effectively removed. Given a regular stormwater inflow (i.e. wet periods), *E. coli* removal by biofilters was about 2 log, which is consistent with the reported performance of similar systems (Hathaway et al., 2009; Zhang et al., 2011). Intermittent dry periods, however, had a significant negative effect on *E. coli* removal (0.6 log) by vegetated biofilters.

A saturated zone with carbon source at the base of stormwater biofilters seems to be beneficial for eliminating the negative effect of dry periods on the removal of *E. coli*. The mean effluent *E. coli* concentrations from biofilters with saturated zone and carbon source were below the guideline value for secondary contact recreational water use. The presence of a saturated zone in biofilters has also been shown to enhance the removal of nitrogen (Dietz and Clausen, 2006) and heavy metals (Blecken et al., 2009a) from stormwater. This configuration, however, is not universally beneficial. F-RNA coliphages, for example, was removed to a lesser degree in those biofilters after dry periods, possibly because it provided a suitable environment for survival. Future work should focus on verifying the effectiveness of this configuration on reference viral pathogen removal, instead of on indicator viruses.

The results of this study suggest that stormwater biofiltration systems, constructed using current design approaches, are incapable of providing reliable removal of protozoa, bacteria, or viruses for safe stormwater discharge or harvesting. Depending on the designated end-use, designers of stormwater management systems need to consider additional treatment technologies, either pre- or post-storage, where inflows have been treated only by a stormwater biofilter and the water is to be used or stored in such a way that a risk to human health exists. Post-storage disinfection, sourcing the wide range of packaged treatment technologies (e.g. ozone, UV or chlorine-injection) available, may be required. It is also evident that a significant research imperative is warranted by the refinement of stormwater biofiltration technologies, using alternative media, to improve their ability to deliver consistent pathogen treatment to the standards required for stormwater discharge or harvesting (Li et al., 2011).

5. Conclusions

During wet periods i.e. regular stormwater inflow, all biofilter designs performed similarly for the removal of indicator organisms from stormwater: the level of *C. perfringens* spores and F-RNA coliphages in stormwater was reduced to below method detection limits (i.e. *C. perfringens* 10 cfu/100 ml, and F-RNA coliphages 1 pfu/100 ml), but only 2 log reduction of *E. coli* was achieved.

Intermittent dry periods of 2 weeks or more had no obvious effect on the removal of *C. perfringens* spores and F-RNA coliphages, except for the columns with a saturated zone, which showed reduced removal efficiency for F-RNA coliphages after dry periods.

E. coli removal by vegetated biofilters, however, became significantly worse after dry periods. The introduction of a saturated zone and carbon source into biofilters completely eliminated the negative effect of drying on *E. coli* removal.

Biofilters with a saturated zone could remove *E. coli* to the recommended water quality for secondary contact recreational water use. Their effectiveness, however, must be validated for reference pathogens.

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